



UNIVERSITI PUTRA MALAYSIA

**MOLECULAR ANALYSIS OF *DICHELOBACTER NODOSUS*
ISOLATED FROM FOOTROT INFECTED SHEEP IN MALAYSIA**

ZUNITA BINTI ZAKARIA

FPV 2001 7

**MOLECULAR ANALYSIS OF *DICHELOBACTER NODOSUS*
ISOLATED FROM FOOTROT INFECTED SHEEP IN MALAYSIA**

By

ZUNITA BINTI ZAKARIA

**Thesis Submitted in Fulfilment of the Requirement for the Degree of
Doctor of Philosophy in the Faculty of Veterinary Medicine
Universiti Putra Malaysia**

December 2001



Abstract of thesis presented to the Senate of Universiti Putra Malaysia
in fulfilment of the requirement for the degree of Doctor of Philosophy

**MOLECULAR ANALYSIS OF *DICHELOBACTER NODOSUS*
ISOLATED FROM FOOTROT INFECTED SHEEP IN MALAYSIA**

By

ZUNITA BTE ZAKARIA

December 2001

Chairman: Professor Dato' Dr. Sheikh Omar Abdul Rahman
B.V.Sc. (Queensland), M.V.Sc. (Saskatchewan),
M.R.C.V.S. (London)

Faculty: Veterinary Medicine

Footrot has become an increasingly important disease of sheep in Malaysia. Therefore, the molecular analysis of the causative agent of footrot, *Dichelobacter nodosus* isolated from footrot infected sheep was undertaken. Fifteen *D. nodosus* isolates were recovered from 38 sheep showing clinical signs of footrot in two government sheep farms located approximately 200 km apart. The isolates were studied and results analysed. Preliminary identification of the organism was carried out by the Gram-stain method while the polymerase chain reaction (PCR) method using species-specific

primers, A and Ac, was employed for species confirmation. All 15 isolates produced a single product of approximately 780 basepairs. Although obtained from two different locations, all isolates were found to be of serogroup B. Two conventional methods, namely the elastase and gelatin-gel tests, were used to assess the virulence of the isolates. Generally, the isolates exhibited variations in the laboratory characteristics. Based on the virulence assessment, some of the isolates appeared to have the capability for causing virulent footrot but were isolated from sheep that did not show clinical signs of the virulent form of footrot. This was probably due to the constant topical treatment regime and the vaccination programme practised by the farm management which may have caused the bacteria to not fully express its virulence characteristics.

Analysis of the fimbrial subunit gene sequence revealed the local strains had sequences that are distinct from the prototype strains. There were 94 to 97 percent amino acid similarities (identities and conserved changes) between the local isolates and the prototype strains. The expression of *D. nodosus* fimbriae serotype B2 in an easily grown aerobe, *Pseudomonas aeruginosa*, were carried out successfully. *Dichelobacter nodosus* fimbrial subunit gene was cloned in an expression vector, pUCpKS downstream the *lac* promoter to construct the recombinant plasmid

pMAL99. Recombinant *P. aeruginosa* cells containing this construct were able to produce a high yield of fimbriae. The fimbriae were physically, structurally and antigenically indistinguishable from those produced by the *D. nodosus* isolates from which the fimbrial subunit gene was originally derived. This was shown and confirmed by Western blot analysis. When the fimbriae produced by the *P. aeruginosa* harbouring pMAL99 were extracted, purified and used as vaccines in sheep, the results conclusively showed that these vaccines were equally effective as either the native whole cells or isolated fimbriae from *D. nodosus* in eliciting the antibody response. The vaccinated sheep were found protected against homologous serogroup challenge. The recombinant fimbriae also produced cross-protective antibodies to heterologous serotypes B3 and B4 infections. Therefore, the monovalent serogroup specific recombinant vaccine has a good potential for use in farms in this country to protect sheep against footrot.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra
Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**ANALISIS MOLEKUL *DICHELOBACTER NODOSUS* YANG
DIPENCILKAN DARI BEBIRI YANG MENGHIDAP REPUT KAKI DI
MALAYSIA**

Oleh

ZUNITA BTE ZAKARIA

Disember 2001

Pengerusi: Profesor Dato' Dr. Sheikh Omar Abdul Rahman
B.V.Sc. (Queensland), M.V.Sc. (Saskatchewan),
M.R.C.V.S. (London)

Fakulti: Perubatan Veterinar

Penyakit reput kaki adalah penyakit yang semakin penting di Malaysia. Oleh itu, analisis molekul ke atas bakteria penyebab penyakit reput kaki, *Dichelobacter nodosus* yang dipencilkan dari bebiri yang menghidap penyakit reput kaki telah dijalankan. Lima belas isolat *Dichelobacter nodosus* telah dipencilkan dari 38 ekor bebiri yang menghidap penyakit reput kaki dari dua ladang kerajaan yang terletak lebih kurang 200 km di antara satu sama lain. Pencilan tersebut dikaji dan dianalisis. Diagnosis telah berjaya dilakukan melalui kaedah pewarnaan Gram sementara reaksi

polimerase rantai (PCR) dengan menggunakan primer spesifik, A dan Ac telah digunakan untuk konfirmasi spesies. Kesemua isolat didapati menunjukkan reaksi positif dalam kaedah PCR dengan menghasilkan satu produk 780 pasangan bes. Walaupun semua isolat dipencilkan dari dua lokasi yang berbeza, semuanya adalah dari satu serogroup B. Dua kaedah konvensional iaitu, ujian elastase dan gel-gelatin telah digunakan untuk menentukan tahap kevirulenan isolat. Secara amnya, isolat menunjukkan variasi dalam ciri-ciri kevirulenan di dalam makmal. Seseengah isolat didapati mempunyai keupayaan menyebabkan tahap penyakit yang virulen, tidak menunjukkan tanda klinikal yang sedemikian. Ini mungkin disebabkan oleh rawatan topikal yang diamalkan oleh pihak pengurusan ladang dan mungkin juga hasil dari program vaksinasi yang menyebabkan bakteria tidak menunjukkan tahap kevirulenan sebenar.

Analisis jujukan gen fimbria menunjukkan isolat tempatan mempunyai jujukan yang berbeza dari strain prototaip. Terdapat 94 hingga 97 peratus persamaan (identiti dan penggantian konserve). Ekspresi fimbria serotip B2 *D. nodosus* di dalam bakteria aerobik, *Pseudomonas aeruginosa* telah berjaya dilakukan. Gen fimbria *D. nodosus* telah diklonkan ke dalam vector ekspresi, pUCpKS selepas kedudukan promoter *lac* untuk membentuk plasmid rekombinan pMAL99. Sel-sel rekombinan *P. aeruginosa* yang

mengandung plasmid ini didapati menghasilkan fimbria yang sama dari segi fizikal, struktur and antigenisiti dengan fimbria asal *D. nodosus*. Ini telah ditunjukkan dan dibuktikan dengan analisis pemblotan Western. Fimbria yang dihasilkan dari *P. aeruginosa* yang membawa pMAL99 diekstrak, ditulenkan dan digunakan sebagai vaksin kepada bebiri. Keputusan menunjukkan vaksin ini mempunyai tahap efektif yang setara dengan sel penuh *D. nodosus* ataupun fimbria yang dipencilkan dari *D. nodosus* itu sendiri dalam menginduksikan respons antibodi. Bebiri yang divaksinasi adalah dilindungi dari cabaran dari serogroup homologus. Fimbria rekombinan ini juga didapati menghasilkan perlindungan silang kepada infeksi dari serogroup heterologus B3 dan B4. Oleh itu, terdapat potensi yang baik untuk mengaplikasi vaksin serogroup spesifik di ladang tempatan untuk melindungi bebiri daripada penyakit reput kaki.

ACKNOWLEDGEMENTS

In the name of Allah, The Most Gracious, Most Merciful, I am thankful for giving me the strength, which has enabled me to complete this study.

I am especially grateful to my supervisor, *Professor Dato' Dr. Sheikh Omar Abdul Rahman* for his invaluable advice, support and guidance. His encouragement and unbounded optimism has always kept my spirits high. I am also very grateful to *Dr. Abdul Rahim Mutalib*, who has spent so much time and effort to improve the quality of the thesis. Thanks also to *Associate Prof. Dr. Mohd. Azmi Mohd. Lila* and *Assoc. Prof. Dr. Son Radu* for their invaluable comments and suggestions throughout the duration of the study.

I thank the *Department of Veterinary Services* which kindly provided samples for this project and to *Professor J.S. Mattick* and *Dr. C. Whitchurch* of The University of Queensland and *SEARCA* for their help in enabling me to attend a training course on molecular biology in Australia. I also thank The Ministry of Science, Technology and



Environment, Malaysia for financial assistance through IRPA grants no. 51011 and 51488.

Special thanks to my husband, *Hasnal Hashim* whom I owe the most for his patience, understanding and support through this long and demanding project. I also express my greatest appreciation to my parents who have always encouraged me to reach new heights I also extend my deepest love to my son, *Harith Zahrin*.

I am also indebted to all my friends especially *Dr. Karim Al-Jashamy* who helped me a lot in handling sheep for some of the experiments, *Dr. Firoz Mian* and *Dr. Siti Khairani Bejo* who were always there to lend a helping hand.

Finally, thanks to all the academic and support staffs of the Faculty of Veterinary Medicine for their help and support during the course of the project.

I certify that an Examination Committee met on 12th December 2001 to conduct the final examination of Zunita binti Zakaria on her Doctor of Philosophy thesis entitled "Molecular Analysis of *Dichelobacter nodosus* Isolated from Footrot Infected Sheep in Malaysia" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Abdul Rani Bahaman, Ph.D.
Professor,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Dato' Sheikh Omar Abdul Rahman, MVSc
Professor,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Supervisor)

Abdul Rahim Mutalib, Ph.D.
Lecturer,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Mohd. Azmi Mohd. Lila, Ph.D.
Associate Professor,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Son Radu, Ph.D.
Associate Professor,
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

John Egerton, Ph.D.
Faculty of Veterinary Science
The University of Sydney
(External Examiner)



AINI IDERIS, Ph.D.
Professor/Dean of Graduate School
Universiti Putra Malaysia

Date: 8 JAN 2002

This thesis submitted to Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy.

AINI IDERIS, Ph.D.
Professor,
Dean of Graduate School
Universiti Putra Malaysia

Date:

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



(ZUNITA BINTI ZAKARIA)

Date: 8 / 1 / 2002

TABLE OF CONTENTS

ABSTRACT	ii
ABSTRAK.....	v
ACKNOWLEDGEMENTS.....	viii
APPROVAL SHEET.....	x
DECLARATION SHEET.....	xii
LIST OF TABLES.....	xvii
LIST OF FIGURES.....	xviii
LIST OF NOTATION.....	xx

CHAPTER

I	GENERAL INTRODUCTION.....	1
II	LITERATURE REVIEW.....	5
	Introduction.....	5
	Characteristics of <i>Dichelobacter nodosus</i>	6
	Taxonomy.....	6
	Morphological and Bacteriological	
	Characteristics of <i>D. nodosus</i>	8
	Nutrition and Energy Metabolism.....	9
	Ultrastructure.....	9
	Serological Classification.....	11
	Molecular Biology of the Fimbriae of	
	<i>D. nodosus</i>	14
	Footrot Disease.....	17
	Historical Background.....	17
	Clinical Characteristics of Ovine Footrot	17
	Laboratory Characteristics.....	20
	Virulence Assessment	21
	Pathogenesis.....	25
	Predisposing Factors.....	26
	Transmission.....	27
	Antibody Responses of Sheep to Footrot	28
	Control and Treatment.....	29
	Footrot in Other Species.....	38
	Footrot in Malaysia.....	39



III	ISOLATION, IDENTIFICATION AND VIRULENCE	
	ASSESSMENT OF <i>Dichelobacter nodosus</i>	47
	Introduction.....	47
	Materials and Methods	
	Sampling Area.....	50
	Sampling Procedure.....	50
	Direct Smear Identification.....	51
	Preparation of Ground Ovine	
	Hoof Horn.....	51
	Preparation of Hoof Agar (HA).....	51
	Trypticase Arginine Serine (TAS) broth.....	52
	Isolation.....	53
	Identification and Confirmation of Suspected	
	<i>D. nodosus</i> Isolate by Polymerase Chain	
	Reaction.....	53
	Maintenance and Preservation of	
	Cultures.....	56
	Serological Grouping.....	59
	Virulence Assessment.....	61
	Results	
	Clinical Observation.....	65
	Direct Smear Examination.....	66
	Identification and Species Confirmation.....	67
	Serogrouping and Serotyping.....	67
	Virulence Test.....	68
	Discussion.....	69
IV	SEQUENCING AND ANALYSIS OF FIMBRIAL	
	SUBUNIT GENE OF LOCAL <i>Dichelobacter nodosus</i>	
	ISOLATES.....	84
	Introduction.....	84
	Materials and Methods	
	Bacteria	86
	Vector.....	86
	Fimbrial Subunit Gene Amplification	
	and Cloning.....	86
	Purification of the PCR Product	87
	Cloning of the PCR Product.....	88
	Screening of Clones.....	89
	Restriction Enzyme Digestion	90
	Sequencing of the Fimbrial Subunit Gene.....	90

	Small Scale Isolation of Plasmid DNA	93
	Preparation of <i>E. coli</i> DH5 α Competent Cells Using Calcium Chloride.....	94
	Results	
	Fimbrial Gene Amplification and Cloning into pGEM-T Easy System.....	95
	Sequence Analysis of Fimbrial Subunit Gene...	96
	Discussion.....	99
V	MORPHOGENETIC EXPRESSION OF LOCAL <i>Dichelobacter nodosus</i> FIMBRIAL SUBUNIT GENE IN <i>Pseudomonas aeruginosa</i>	111
	Introduction.....	111
	Materials and Methods	
	Bacterial Strain.....	113
	Vectors.....	114
	Fimbrial Subunit Gene Amplification and Cloning.....	114
	Digestion of Recombinant pGEM-T Plasmid and pUC PKS.....	115
	Recloning of the Fimbrial Subunit Gene.....	115
	Surface Fimbriae Preparation.....	118
	Polyacrylamide Gel Electrophoresis.....	119
	Western Blotting.....	120
	Preparation of <i>Pseudomonas aeruginosa</i> Competent Cells.....	123
	Results.....	124
	Discussion.....	126
VI	IMMUNOGENIC PROPERTIES OF SEROGROUP SPECIFIC RECOMBINANT VACCINE IN SHEEP.....	137
	Introduction.....	137
	Materials and Methods.....	139
	<i>Experiment I: Antigenic Response Of</i> Local Vaccine In Rabbits.....	139
	Experimental Design.....	139
	Collection of Serum.....	139
	<i>Experiment II: Antigenic Response of</i> Local Vaccine in Sheep.....	140
	Sheep.....	140
	Experimental Animal Enclosure.....	140

Preparation Of Recombinant <i>D. nodosus</i>	
Fimbriae Vaccine.....	141
Preparation Of <i>D. nodosus</i> Whole Cells Vaccine..	141
Preparation Of Purified <i>D. nodosus</i> Fimbriae	
Vaccine.....	142
Collection of Serum.....	142
Experimental Design.....	143
Assessment of Lesions.....	145
Serum Antibody Responses.....	145
Results	
Serum Antibody Response in Rabbits.....	146
Assessment of Lesions in Sheep.....	147
Serum Antibody Response in Sheep.....	148
Discussion.....	149
VII GENERAL DISCUSSION AND CONCLUSION.....	159
General Discussion.....	159
Future Direction.....	170
Conclusion.....	171
BIBLIOGRAPHY.....	173
APPENDICES.....	194
Appendix A: Definition of scoring system.....	194
Appendix B: Gram stain- Kopeloff's Modification..	195
Appendix C1: Common Solutions.....	196
Appendix C2: Solutions for SDS-PAGE.....	198
Appendix C3: Solutions for Western Blotting.....	200
Appendix D1: Nucleotide Sequences of the fimbrial	
Subunit gene of N1 to N15.....	201
Appendix D2: Alignment of amino acid sequences	
Predicted from the <i>fimA</i> subunit gene of isolates	
N1 to N15 and <i>D. nodosus</i> serogroup B prototypes.	204
Appendix E1: Vector pGEM-T.....	206
Appendix E2: Vector pUCpKS.....	206
Appendix F: Construction of pJSM202.....	207
BIODATA.....	208

LIST OF TABLES

Table	Page
2.1 Biochemical properties of <i>Dichelobacter nodosus</i>	42
2.2 Serogroups of <i>Dichelobacter nodosus</i> determined by cross-tube agglutination test.....	43
2.3 Percent identity in sequence comparisons of fimbriae from <i>D. nodosus</i> strains of different serogroups.....	44
3.1 Agglutination titre reaction between <i>Dichelobacter nodosus</i> antigen and serotype specific antisera.....	80
3.2 Comparative examination of 15 <i>D. nodosus</i> isolates using elastase and gelatin-gel tests.....	81
4.1 Amino acid similarities in <i>D. nodosus</i> isolated in Malaysia	108
6.1 Footscore of individual sheep in control group at days after artificial infection with strain N3.....	155
6.2 Agglutination titres in serum of vaccinated sheep with various vaccine preparation from day 0 to day 72 experiment against homologous antigen.....	156
6.3 Mean agglutination titres in serum of vaccinated sheep with recombinant fimbriae at 5 weeks post booster against serotype B3 and serotype B4 antigen.....	157



LIST OF FIGURES

Figure		Page
2.1	Schematic representation of <i>D. nodosus</i> ultrastructure as determined by the negative staining and thin section electron microscopy techniques.....	45
2.2	Diagrammatic representation of <i>D. nodosus</i> fimbriae sequences to illustrate the disposition of disulphide bridges and homologous N-terminal and C-terminal sequences.....	46
3.1	A Gram-stained smear from a footrot lesion showing a bacilli resembling <i>D. nodosus</i> seen as a large Gram negative rod with swollen ends.....	82
3.2	A 2% HA showing colony morphology of <i>D. nodosus</i>	82
3.3	Cells from the colonies showed typical large Gram-negative straight curved rod with bulb ends.....	83
3.4	Agarose gel electrophoresis of <i>D. nodosus</i> genomic DNA amplification products using Ac and C primer combination.....	83
4.1	Agarose gel electrophoresis of <i>D. nodosus</i> genomic DNA amplification products using PTC5 and PTC830 primer combination.....	109
4.2	Plasmid extraction of clones.....	109
4.3	Digestion of plasmid extracted from positive clones.....	110
4.4	103 conserved nucleotide sequence upstream the initiation codon of <i>fimA</i> gene of <i>D. nodosus</i>	110
5.1	<i>SacI</i> and <i>ApaI</i> digestion of pUCpKS and pGEM-T+insert.	134

Figure		Page
5.2	Digestion of plasmid extracted from positive clones.....	134
5.3	SDS-PAGE of purified fimbriae extracted from wild type PAK <i>P. aeruginosa</i> and recombinant PAK <i>P. aeruginosa</i>	135
5.4	Western blot of fimbriae isolated from wild type PAK <i>P.</i> <i>aeruginosa</i> and recombinant PAK <i>P. aeruginosa</i>	135
5.5	Construction and genealogy of pMAL99 for high level production of <i>D. nodosus</i> fimbriae in <i>P. aeruginosa</i>	136
6.1	Infected foot with lesion score 3.....	158
6.2	Infected foot with lesion score 4.....	158

LIST OF NOTATION

Amino acid	one-letter notation
alanine	A
arginine	R
asparagine	N
aspartate	D
cysteine	C
glutamate	E
glutamine	Q
glycine	G
histidine	H
isoleucine	I
leucine	L
lysine	K
methionine	M
phenylalanine	F
proline	P
serine	S
threonine	T
tryptophan	W
tyrosine	Y
valine	V

CHAPTER 1

GENERAL INTRODUCTION

Footrot is a contagious disease of ruminants, particularly sheep and goats although cattle and deer may also be affected. It is present worldwide and has a significant economic impact in sheep farming countries with a temperate climate and a moderate to high rainfall, such as Australia and New Zealand (Stewart, 1989). It is responsible for serious losses to the sheep industry in reduced meat and wool production, lowered fertility, and the high costs of labour and materials used in treating affected animals (Stewart *et al.*, 1984; Marshall *et al.*, 1991; Glynn, 1993). The disease is characterized by inflammation of the interdigital skin and hoof matrix leading to an underrunning and separation of the hoof from the epidermal tissues. Classical signs of footrot are severe lameness and pain with the infected animal preferring to walk on its knees when only the front feet are affected, or lying prone when all four feet are affected (White, 1991). The increasing awareness of animal welfare issues associated with footrot has brought the disease more into the forefront of the industry.

The main etiological agent of footrot is *Dichelobacter nodosus* formerly known as *Bacteroides nodosus* (Dewhirst *et al.*, 1990). It is a Gram-negative, strictly anaerobic, nonsporeforming bacteria. A culture of *D. nodosus* deposited in the American Type Culture Collection as accession no. 25549 has been designated the prototype strain (Skerman, 1989). *Dichelobacter nodosus* lives only in diseased hooves and survives no longer than 7-14 days in faeces, soil or pasture.

Footrot has become an increasingly important disease of sheep in Malaysia. Even though the disease is known to exist for the last two centuries in many parts of the world, it has only been detected in this country in the last six years (Yii, 1995). The first confirmed case of footrot in Malaysia occurred in a government sheep farm namely the Institut Haiwan Kluang, Johor; in the southern part of Peninsular Malaysia in 1994. The disease is now present in other farms throughout the country. Importations of sheep were made from diverse countries such as Australia, Brazil and Thailand which might have brought the disease into the country.

Vaccines containing representative strains from all major serogroups incorporated into oil-based adjuvant have been available since 1981. They consist of a mixture of killed cultures of different serogroups (Walker, 1988). The conventional vaccines are very costly (Mattick

and Hobbs, 1990), thus, a genetically engineered vaccine was developed. The fimbrial gene from prototype *D. nodosus* strains has been cloned and expressed as extracellular fimbriae in an easily grown aerobe, *Pseudomonas aeruginosa*. Fimbriae extracted from the recombinant *P. aeruginosa* cells were used as vaccines against *D. nodosus* infection.

Sheep farms in Malaysia started to use the commercial footrot multivalent killed whole cells vaccines in late 1996. The vaccination did reduce the prevalence of footrot but the strength and duration of the immunity achieved was limited. The short duration of the immunity is associated with a poor agglutinin response in animals receiving multiple fimbrial antigens of *D. nodosus* simultaneously. Moreover, a previous study on local *D. nodosus* isolates found them to be antigenically different from the prototype strain (Zunita, 1998). There is now a recurrence of the disease which has become worse under conditions favouring the infection.

Therefore an extensive study is needed to obtain a comprehensive knowledge about the etiological agent and to understand the situation of the infection and the disease in the country. Conventional eradication methods practised in the temperate countries are unlikely

to succeed in Malaysia where the weather is warm and humid throughout the year. The findings of the study will enable the formulation of suitable measures to control footrot.

The objectives of this study were:

- (1) to isolate, identify and assess the virulence of *D. nodosus* from clinical cases of footrot in sheep kept in farms in Malaysia.
- (2) to determine the relationship between fimbriae from different strains in Malaysia and the prototype strains by analysing the nucleotide as well as the amino acid sequences of the fimbrial subunit gene.
- (3) to determine the possibility of producing a serogroup specific recombinant vaccine by cloning and expressing local *D. nodosus* fimbrial subunit gene in an aerobe surrogate host, *Pseudomonas aeruginosa*.
- (4) to investigate the antigenic properties of a recombinant vaccine prepared from fimbrial genes of a local isolate in sheep.